Original Research Paper

Title: Fertilizability of oocytes derived from Holstein cows having different antral follicle counts in ovaries

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Abstract

In this study, to clarify the relationship between ovarian reserve and oocyte quality, cumulus-oocyte complexes (COCs) were collected repeatedly by ovum pick-up (OPU) from cows with high and low antral follicle counts (AFCs) at short (3-4 days) and long (7 days) intervals, and COC morphologies and oocyte fertilizability were examined. The relationship between AFC and follicular growth after OPU was also investigated. Cows showing AFC of ≥ 30 in at least one OPU session were grouped into the high-AFC group. At a short interval, follicular sizes and COC morphologies were similar between the different AFC groups. However, the normal fertilization rate was higher in the high-AFC group than in the low one, although total penetration rates were similar. At a long interval, the percentage of COCs with poor morphology in the high-AFC group was higher and the normal fertilization rate was lower than in the low one. In the low-AFC group, normal fertilization rates at short and long intervals were similar, and mean follicular size became larger at a long than at a short interval. However, mean follicular sizes at short- and long-interval OPU were similar in the high-AFC group. In conclusion, it is suggested that oocytes derived from cows with high AFC had higher fertilizability than those from cows with low AFC when OPUs were performed at a short (3-4 days) interval. However, oocyte quality in high-AFC cows was impaired by long-interval (7 days) OPU, possibly due to the degradation of follicles.

Keywords: Dairy cattle; Ovarian reserve; Antral follicle count; Ovum pick-up; Oocyte quality

37 **Introduction**

38 The constant decline in fertility of dairy cattle has been a problem globally for the last few
39 decades. The conception rate of first insemination after parturition declined from 53.4% (1989) to 41.2%
40 (2008) in Japan (Dochi et al., 2010) and the non-return rate at 70 days after breeding declined from 54%
41 (1996) to 45% (2007) in the United States (Norman et al., 2009). Many researchers focused on nutrition,
42 genetic improvement, and endometritis as candidate factors causing this decline of fertility and investigated
43 them (Grummer et al., 2007; Hansen, 2000; Sheldon et al., 2009), but the fertility of dairy cattle could not
44 be returned to its previous level. Fertility is a multi-factorial trait and its decline has been caused by a
45 network of genetic, environmental, and managerial factors. Their complex interactions make it difficult to
46 determine the exact reason for this decline (Walsh et al., 2010).

47 Recently, ovarian reserve was proposed as a factor in the fertility of mammalian species
48 including dairy cattle (Ireland et al., 2011). Ovarian reserve is defined as the capability of ovaries to
49 produce fertilizable and developmental oocytes resulting in successive conception, and to secrete sex
50 steroid hormones that induce the estrous cycle correctly and sustain pregnancy (te Velde and Pearson, 2002).
51 It was reported that ovarian reserve was influenced by genetic and managerial factors, for example,
52 heredity of early menopause (de Bruin et al., 2001), gene association with premature ovarian failure in
53 humans (Prakash et al., 2010), and maternal undernutrition during the first trimester of the gestation period
54 in ewes (Rae et al., 2001) and cows (Mossa et al., 2013). The number of small antral follicles in ovaries
55 detected by ultrasonography (antral follicle count; AFC) is considered as an indicator of ovarian reserve
56 (Burns et al., 2005) because it reflects the number of primordial follicles in cattle ovaries (Ireland et al.,

2008), and it is stable in individual animals at any day in an estrous cycle (Alvarez et al., 2000; Cushman et al., 2009). It was also reported that dairy cows with low AFC tended to have a long open period, low steroidogenic capability, and poor responsiveness to superstimulatory treatments (Mossa et al., 2012; Jimenez-Krassel et al., 2009; Ireland et al., 2007). However, the relationship between ovarian reserve and the quality of oocytes in cattle is still unclear, even though a reduced ovarian reserve has been linked to aging in humans associated with a reduced quality of oocytes (Eichenlaub-Ritter et al., 2004).

Ireland et al. (2007) reported that the developmental competences of bovine oocytes after *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) were similar regardless of AFC. In this study, oocytes were collected from slaughterhouse materials without considering the estrous cycle of animals, although it is well known that the estrous cycle and follicular size affect the quality of oocytes (Lonergan et al., 1994; Nagano et al., 2007). Therefore, oocytes should be collected at the same stage of the estrous cycle and follicular wave when evaluating oocyte quality. Ultrasound (US)-guided ovum pick-up (OPU) enables repeatable collections of oocytes from growing antral follicles because the ablation of follicles induces the recruitment of new follicular waves (Bergfelt et al., 1994). Therefore, if OPU is performed at the same interval, oocytes will be collected from follicles at the same status repeatedly. Silva-Santos et al. (2014) reported that OPU combined with the *in vitro* production (IVP) of embryos resulted in a larger number of transferable embryos per session from *Bos indicus* × *B. taurus* hybrid females in a high-AFC group (≥40) than in a low-AFC group (≤10). However, the intervals between OPU sessions were random in that study. Hagemann et al. (1999) reported that oocytes with high developmental competence were collected from follicles at the growth phase (2 and 10 days after estrus) compared with those at the dominant phase (7 and

15 days after estrus) derived from ovaries of slaughtered dairy cows. However, AFCs in cows were not considered in that study.

There is also a possibility that the dynamics of follicular development differs between cows with different AFCs because an inverse correlation between AFC and the concentration of follicle-stimulating hormone (FSH) in serum has been reported (Burns et al., 2005; Ireland et al., 2007). Thus, we also need to examine follicular growth after OPU when considering the optimal interval of OPU for cows with different AFCs and its effect on the quality of oocytes.

In the present study, to clarify the relationship between AFC and oocyte quality in cattle, we collected cumulus-oocyte complexes (COCs) by US-guided OPU from cows in which the follicular wave was synchronized, and examined the morphologies of retrieved COCs. It is well known that blastocyst development is markedly affected by the number of oocytes cultured in group (Carolan et al., 1996; Ward et al., 2000). From the low-AFC cows, the number of oocytes collected is limited; therefore, the fertilizability of oocytes after IVM and IVF was evaluated. We also investigated the relationship between AFC and follicular growth after OPU.

Materials & Methods

1. Animals

This study was approved by the Institutional Animal Care and Use Committee of Hokkaido University and Rakuno Gakuen University. The cows were kept at the experimental farms of Hokkaido

University (n = 6; 3 lactating and 3 dry cows) and the Faculty of Veterinary Medicine of Rakuno Gakuen University (n = 8; all dry cows). Their age and parity at Hokkaido University were 7.5 ± 2.9 (mean ± standard deviation) and 4.3 ± 1.9, respectively. The age of the animals at Rakuno Gakuen University was 6.3 ± 1.2; however, their parity was unknown. US-guided OPU was carried out at 2 different intervals, namely, twice a week (3- or 4-day interval: short interval) and once a week (7-day interval: long interval) from July 2013 to June 2014. At Rakuno Gakuen University, 4 dry cows were used for short-interval OPU for 7 weeks from July to August (2, 4, 10 and 13 sessions), and 2 dry cows were used for long-interval OPU for 8 weeks from November to December (8 sessions each). Two dry cows were used for both long (8 sessions each) and short intervals (13 sessions each). At Hokkaido University, 2 dry cows were used for short-interval OPU for 5 weeks from January to February (9 sessions each), and 1 dry (7 sessions) and 3 lactating cows (5, 5 and 7 sessions) were used for long-interval OPU for 7 weeks from May and June. In total, 16 Holstein cows were used in this experiment.

2. Follicle aspiration system

A single-lumen needle (17 gauge, 490 mm long; Misawa Medical, Ibaraki, Japan) was connected to a 50-mL collection tube (Falcon 2070; Becton Dickinson, Franklin Lakes, NJ, USA) via a silicone tube (100 cm long, 1 mm internal diameter). The collection tube was warmed at 37˚C in a portable incubator (FV-5; Fujihira Industry, Tokyo, Japan) and the other silicone tube was connected to a vacuum pump with a foot-pedal switch (K-MAR-5000; Cook Medical Technology, Brisbane, Australia). US-guided OPU was conducted using an ultrasound machine (HS-1500; Honda Electronics, Aichi, Japan) equipped with a

9.0-MHz long-handled micro-convex probe (HCV-3710MV; Honda Electronics), and the number of aspirated follicles was noted. Some OPU sessions were recorded using a digital video recorder connected to the US machine and the diameters of aspirated follicles were measured on a personal computer (56 out of 129 sessions). The diameters of follicles were calculated by halving the summed values for the follicular length of long and short axes. Antral follicles were divided into 3 categories according to their diameters (small: <4 mm, intermediate: 4 - <8 mm, and large: ≥8 mm) because follicles of ≥4 mm in diameter are usually defined as representing the emergence of follicles (Ginther et al., 1989) and follicles of ≥8 mm in diameter start to express luteinizing hormone (LH) receptors (Bao et al., 1997).

3. COC collection and classification

US-guided OPU was performed as previously described by Sasamoto et al. (2003; 2004). Before starting the experiments, follicle ablation under US guidance was carried out for synchronization of the emergence of the follicular wave (Bergfelt et al., 1994). Preceding follicle aspiration, cows were fixed in a treatment stall and injected with 2-4 mL of 2% lidocaine hydrochloride (Xylocine; AstraZeneca, Osaka, Japan) for epidural anesthesia. A long-handled micro-convex probe was inserted into the vagina after cleaning the vaginal region. The circuit from the tip of the aspiration needle to the collection tube was filled with flushing medium to avoid the attachment of contents within follicles to the inner surface and blood coagulation. Flushing medium consisted of Dulbecco's phosphate-buffered saline (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 1% calf serum (Invitrogen, Grand Island, NY, USA), 100 μg/mL streptomycin sulfate (Meiji Seika, Tokyo, Japan), 100 units/mL penicillin G potassium (Meiji Seika Pharma,

137 Tokyo, Japan), and 10 IU/mL heparin sodium (Ajinomoto Pharmaceuticals, Tokyo, Japan). All follicles
138 detected by ultrasonography were counted and aspirated with 100 mmHg vacuum pressure (aspiration flow
139 rate: 16.5 mL/min) as previously reported (Imai et al., 2006; Matoba et al., 2014). The recovered contents
140 within follicles were poured through an EmCon filter (Immuno Systems, Spring Valley, WI, USA) and the
141 filter was rinsed with about 200 mL of flushing medium without heparin. After rinsing, the contents of the
142 filter cup were poured into plastic dishes (Falcon 351005, Becton Dickinson, Franklin Lakes, NJ, USA) and
143 COCs were detected under a stereomicroscope. Retrieved COCs were examined for their morphology
144 under a stereomicroscope and divided into 4 grades: I) oocytes with several compact cumulus layers, II)
145 oocytes denuded partially, III) oocytes denuded completely, and IV) oocytes with expanded cumulus layers,
146 as described previously (Merton et al., 2003).
147
148 *4. In vitro maturation and fertilization*
149 Unless stated otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).
150 All of the collected COCs were matured individually as described previously (Nagano et al., 2013), then
151 COCs from each animal were pooled and inseminated *in vitro* as described previously (Takahashi and
152 Kanagawa, 1998). Briefly, each COC was cultured individually in a well of multi-well plates (Nunc 163118
153 MINI TRAYS; Thermo Fisher Scientific, Roskilde, Denmark) filled with 6 mL of maturation medium in
154 humidified atmosphere of 5% CO_2 in air at 39°C for 22 h. Maturation medium composed of TCM-199
155 (Invitrogen), 10% fetal calf serum (Invitrogen), 0.2 mM sodium pyruvate, 0.02 units/mL FSH (from
156 porcine pituitary), and 1 μg/mL estradiol-17β (E_2). Then, all of matured COCs from each cow were pooled

and inseminated in a droplet of IVF medium. Briefly, the COCs were co-cultured with spermatozoa (5 × 10^6 cells/mL) in 50-μL droplets (1-21 COCs/droplet) of fertilization medium covered with mineral oil in a humidified atmosphere at 5% CO_2, 5% O_2, and 90% N_2 at 39°C for 18 h. Fertilization medium was modified Brackett and Oliphant (mBO) isotonic medium (Brackett and Oliphant, 1975) supplemented with 2.5 mM theophylline and 3 mg/mL bovine serum albumin. After the thawing of frozen semen from a Holstein bull, motile sperm were separated using a Percoll (GE Healthcare, Pittsburgh, PA, USA) gradient (45% and 90%). After 18 h, all oocytes were denuded by vortexing, fixed with fixative solution (75% ethanol and 25% acetic acid) on glass slides, and stained with 1% aceto-orcein solution, as described previously (Nagano et al., 2006). Fertilization status was examined under a phase contrast microscope as follows: oocytes with male and female pronuclei with a corresponding sperm tail (2PN), oocytes with an enlarged sperm head with an anaphase I or telophase I chromosome (ESH), and oocytes with more than two enlarged sperm heads or male pronuclei (polyspermy). We defined oocytes with 2PN as representing normal fertilization.

5. Experimental design

Antral follicular count was determined in all cows at each OPU session. Cows that showed AFC of ≥30 in at least one session during the experimental period were classified into the high-AFC group. The mean numbers of follicles in the high- and low-AFC groups for all experiments were 26.1 ± 6.1 (ranging from 12 to 50 follicles, n = 61) and 16.9 ± 5.4 (ranging from 7 to 26 follicles, n = 68) ($P < 0.05$), respectively. All follicles detected were subjected to aspiration; however, we sometimes failed to aspirate

some of the small ones because of their positions. The ages of the animals in the high- and low-AFC groups were 6.9 ± 1.8 and 6.6 ± 2.6 years old, respectively. These cows were subjected to OPU at either a short (3 to 4 days) or a long (7 days) interval. The number of retrieved COCs was recorded, and the recovery rate of COCs based on aspirated follicles was calculated. After COC collection, the morphological grade of the retrieved COCs was determined, and all of them were subjected to IVM and IVF. Then, the fertilization status of oocytes was examined.

6. Statistical analysis

Data obtained from dry and lactating cows from 3- and 4-day-interval OPU were combined because of their similarities. The numbers of collected COCs and the recovery rate of COCs in high- and low-AFC groups were compared by two-way ANOVA followed by Student's *t*-test. The data indicated by percentages were analyzed by chi-square test. Mean diameter of follicles was analyzed by Mann-Whitney's U test. All analyses were performed using software (JMP Pro 10.6, SAS Institute Inc., Cary, NC, USA). Values were considered significantly different at $P < 0.05$.

Results

1. Collection of COCs by OPU and their morphologies

There was a significant difference in the mean numbers of follicles between the high- and low-AFC groups at short and long intervals as shown in Table 1. There were interactions between AFC and

OPU interval in the numbers of follicles aspirated and COCs collected ($P < 0.05$), but not in the recovery rate of COCs ($P = 0.28$). The recovery rates of COCs were similar between groups. The number of aspirated follicles was larger in the high-AFC group at each interval. At a long interval, the number of collected COCs was larger in the high-AFC group than in the low-AFC group. As shown in Table 2, when OPU was carried out at a short interval, there was no difference in the morphology of COCs between the high- and low-AFC groups. However, at a long interval, the percentage of grade I COCs in the high-AFC group were lower and the percentage of grade III COCs in the high-AFC group were higher than those in the low-AFC group ($P < 0.05$).

2. Sizes of aspirated follicles at OPU sessions

There were interactions between AFC and OPU interval in the mean number of aspirated follicles and the mean diameter of follicles ($P < 0.05$). As shown in Table 3, mean diameters of aspirated follicles were similar between the high- and low-AFC groups at each OPU interval. At a long interval, the proportion of large-sized follicles in the low-AFC group was greater than that in the high-AFC group ($P < 0.05$).

In the high-AFC group, there were no differences in follicular diameter and proportions of follicles in each diameter between the different OPU intervals. On the other hand, in the low-AFC group, the proportion of large-sized follicles at a long interval was higher and the proportion of small-sized follicles at a long interval was lower than those at a short interval ($P < 0.05$). The mean diameter of follicles was also larger at long interval than at short interval.

3. Fertilizability of oocytes after in vitro maturation and fertilization

As shown in Table 4, at a short interval, total penetration rates were similar between the high- and low-AFC groups; however, the proportion of oocytes having 2PN in the high-AFC group was higher than that in the low-AFC group ($P < 0.05$). At a long interval, both the proportion of oocytes having 2PN and the total penetration rate in the high-AFC group were lower than those in the low-AFC group ($P < 0.05$). In the low-AFC group, there was no difference in fertilization status between different OPU intervals; however, in the high-AFC group, 2PN and total penetration rates at a long interval were lower than those at a short interval ($P < 0.05$).

Discussion

In the present study, we allocated cows into high- and low-AFC groups based on the peak AFC (high: ≥30, low: <30). The mean numbers of follicles in the high- (26.1 ± 6.1) and low-AFC groups (16.9 ± 5.4) in this study were similar to the criteria of bovine ovarian reserve (high: ≥25, low: ≤15 follicles) (Ireland et al., 2011). Most of the OPU sessions were carried out without corpus luteum (CL) because CL in each cow had regressed spontaneously until the second or third OPU session (at least 11 days after the first follicle ablation). Therefore, the timings of OPU performed in the present study correspond to 3.5 to 4.5 (short interval) and 7.5 days (long interval) after estrus because the emergence of a follicular wave occurs approximately 2 days after estrus (Sirois and Fortune, 1988) and 1.5 days after the ablation of follicles

237 (Bergfelt et al., 1994).

238 At a short OPU interval, there were no differences in the distributions of COCs with different
239 morphologies and follicles with different diameters between the high- and low-AFC groups. These results
240 indicate that COCs were collected from follicles at the same phase in the follicular wave. After IVF, the
241 total penetration rates in each group were also similar; however, the normal fertilization rate in the
242 high-AFC group was higher than that in the low one. These results suggest that oocytes derived from cows
243 with high AFC have higher competence for fertilization. In the present study, IVF media did not contain
244 heparin, which induces semen capacitation (Parrish, 2014); thus, cumulus cells might play an important
245 role in the present IVF system. A possible reason for the low fertilizability of oocytes collected at a long
246 interval in the high-AFC group is the low quality of cumulus cells. Hyaluronan synthase 2 (HAS2),
247 gremlin1 (GREM1) and pentraxin 3 (PTX3), genes expressed in cumulus (granulosa) cells are known to be
248 indicators for evaluating the quality of human oocyte (Cillo et al., 2007) and especially HAS2 is also
249 reported to be an indicator for predicting developmental competence of bovine oocyte (Assidi et al., 2008;
250 Salhab et al., 2010). Therefore, we should investigate the quality of cumulus (granulosa) cells derived from
251 different AFC cows. In addition, Iwata et al. (2013) reported that oocytes from older beef cows had a lower
252 mitochondrial DNA copy number than those from younger cows, resulting in lower rates of nuclear
253 maturation, cleavage, and development to blastocyst *in vitro*. Ovarian reserve is considered to decrease
254 according to maternal aging; therefore, the difference in AFC may also be considered to affect the number
255 and activity of mitochondria. The ages of cows with different AFC used in the present study were similar;
256 however, it is necessary to examine mitochondrial DNA copy number and activity of oocytes derived from

257 different AFC cows in further study.

258 It is well known that extending the OPU interval decreases oocyte quality and developmental
259 competence after IVF because of the higher incidence of atretic follicles (Hanenberg and van
260 Wagtendonk-de Leeuw, 1997; Hagemann et al., 1999). It was reported that dominant follicles reach their
261 maximum sizes at 6 to 7 days after estrus (Sirois and Fortune, 1988), and E_2 and inhibin secreted from these
262 follicles suppress the development of other smaller follicles by the inhibition of FSH secretion (Ginther et
263 al., 1996). These suggest the possibility that we collected COCs from follicles before follicular deviation at
264 a short OPU interval, but from follicles after deviation at a long OPU interval. Indeed in the high-AFC
265 group at long OPU interval, the proportion of follicles with different sizes was similar to that at short
266 interval, and the percentages of COCs with low-graded morphology increased and high-graded morphology
267 decreased. In addition, total penetration and normal fertilization rates of oocytes showed lowest values
268 between groups. These results indicate that follicles in the high-AFC group start to degenerate until 7 days
269 after OPU, and support the previous reports (Hanenberg and van Wagtendonk-de Leeuw, 1997; Hagemann
270 et al., 1999). On the other hand, the mean diameter of follicles in the low-AFC group at long interval was
271 larger than that at short interval due to an increase in the proportion of large-sized follicles. In addition,
272 normal fertilization and total penetration rates in the low-AFC group were similar between short and long
273 OPU intervals. These results indicate that follicles in the low-AFC group continue to grow during extended
274 period from 3-4 days to 7 days of OPU. The inverse correlation between AFC and the concentrations of
275 FSH and inhibin in serum was reported in cattle (Burns et al., 2005); namely, low-AFC cows tended to
276 show higher FSH and lower inhibin concentrations than high-AFC ones. Therefore, it is speculated that the

deviation of growing follicles in low-AFC cows occurs later than 7 days after OPU probably due to high FSH concentration.

In conclusion, the oocytes derived from high-AFC cows have higher fertilizability than those from low-AFC cows when OPU is conducted at a 3- to 4-day interval. The difference of follicular growth between high- and low-AFC cows observed in the present study indicates that the deviation of small follicles occurs earlier in the high-AFC group than in the low-AFC group. Therefore, the quality of oocytes derived from high-AFC cows may be impaired by extending the OPU interval to 7 days. Oocyte collection from cows by OPU at a short interval is recommended, especially for high-AFC cows. In further study, embryonic development after IVF should be examined by increasing the number of oocytes cultured in group and also embryo transfer should be performed in order to clarify the relationship between the developmental competence of oocytes to progeny and AFC.

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Table 1. Effects of OPU interval and antral follicle count (AFC) on the collection of cumulus-oocyte complexes (COCs)

OPU Interval	AFC group	No. of cows (No. of sessions)	No. of follicles detected (n)	No. of follicles aspirated (n)	No. of COCs collected (n)	Recovery rate of COCs (%)
Short	High	3 (35)	24.5 ± 6.8[a] (856)	22.6 ± 6.7[a] (792)	6.2 ± 3.3 (217)	28.1 ± 16.6
	Low	5 (38)	17.4 ± 5.7[b] (662)	16.0 ± 5.0[b] (609)	4.7 ± 2.8 (178)	28.6 ± 15.3
Long	High	4 (26)	28.3 ± 4.3[a]* (736)	27.1 ± 4.2[a]* (705)	9.7 ± 3.6[a]* (252)	35.7 ± 12.1
	Low	4 (30)	16.2 ± 4.8[b] (486)	15.7 ± 4.7[b] (472)	4.7 ± 3.4[b] (142)	30.2 ± 19.4

Values are mean ± standard deviation.

[a, b] Different superscripts indicate a significant difference between AFC groups at the same OPU interval.

* Asterisks indicate a significant difference between the same AFC groups at different OPU intervals.

Table 2. Effects of OPU interval and antral follicle count (AFC) on the morphology of cumulus-oocyte complexes (COCs)

OPU Interval	AFC group	No. of cows (No. of sessions)	No. of COCs collected	Proportion (n) of COCs graded as			
				I	II	III	IV
Short	High	3 (35)	217	43.8 (95)	25.8 (56)	27.6 (60)	2.8 (6)
	Low	5 (38)	178	47.1 (84)	23.6 (42)	27.0 (48)	2.2 (4)
Long	High	4 (26)	252	37.7[b] (95)	20.2 (51)	36.5[a]* (92)	5.6 (14)
	Low	4 (30)	142	47.9[a] (68)	21.8 (31)	22.5[b] (32)	7.7 (11)

[a, b] Different superscripts indicate a significant difference between AFC groups at the same OPU interval.

* Asterisks indicate a significant difference between the same AFC groups at different OPU intervals.

Table 3. Effects of OPU interval and antral follicle count (AFC) on aspirated follicular size

OPU Interval	AFC group	No. of cows (sessions)	Mean no. of aspirated follicles (n)	Mean diameter (mm) of follicles (range)	Proportion (n) of follicles of each diameter		
					<4 mm	4 - <8 mm	≥8 mm
Short	High	3 (12)	23.1 ± 4.8[a] (277)	4.5 ± 2.2 (1.6 - 18.4)	54.2 (150)	38.6 (107)	7.2 (20)
	Low	3 (12)	16.7 ± 3.1[b] (200)	4.3 ± 1.9 (1.6 - 11.6)	56.5* (113)	39.0 (78)	4.5 (9)
Long	High	4 (16)	29.8 ± 5.0[a]* (477)	4.7 ± 2.6 (1.8 - 21.4)	50.9 (243)	41.5 (198)	7.5[b] (36)
	Low	4 (16)	14.4 ± 3.5[b] (230)	5.2 ± 3.4* (1.9 - 24.6)	46.5 (107)	41.3 (95)	12.2[a]* (28)

Values are mean ± standard deviation.

[a, b] Different superscripts indicate a significant difference between AFC groups at the same OPU interval.

* Asterisks indicate a significant difference between the same AFC groups at different OPU intervals.

Table 4. Effects of OPU interval and antral follicle count (AFC) on fertilization statuses after *in vitro* maturation and fertilization

OPU Interval	AFC group	No. of COCs (replicates)	Proportion (n) of oocytes with			Proportion (n) of oocytes penetrated by sperm
			2PN	ESH	polyspermy	
Short	High	217 (34)	29.0 [a]* (63)	13.8 (30)	8.3 (18)	51.2* (111)
	Low	178 (35)	19.7[b] (35)	14.6 (26)	10.1 (18)	44.4 (79)
Long	High	252 (26)	8.3[b] (21)	8.7 (22)	4.8 (12)	21.8[b] (55)
	Low	142 (29)	21.3[a] (30)	9.9 (14)	5.0 (7)	36.2[a] (51)

[a, b] Different superscripts indicate a significant difference between AFC groups at the same OPU interval.

* Asterisks indicate a significant difference between the same AFC groups at different OPU intervals.

2PN: male and female pronuclei, ESH: enlarged sperm head with anaphase I or telophase I oocyte.